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# **Adverse Benzo[a]pyrene Effects on Neurodifferentiation Are Altered by Other Neurotoxicant Coexposures: Interactions with Dexamethasone, Chlorpyrifos, or Nicotine in PC12 Cells**

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Abbreviations:

ANOVA, analysis of variance -

BaP, benzo[a]pyrene -

ChAT, choline acetyltransferase -

PAH, polycyclic aromatic hydrocarbon -

TH, tyrosine hydroxylase -

## ABSTRACT

**BACKGROUND:** Polycyclic aromatic hydrocarbons are suspected developmental neurotoxicants but human exposures typically occur in combination with other neurotoxic contaminants.

**OBJECTIVE/METHODS:** We explored the effects of benzo[a]pyrene (BaP) on neurodifferentiation in PC12 cells, in combination with a glucocorticoid (dexamethasone, used in preterm labor), an organophosphate pesticide (chlorpyrifos) or nicotine.

**RESULTS:** By itself, BaP suppressed the transition from cell division to neurodifferentiation, resulting in increased cell numbers at the expense of cell growth, neurite formation and development of dopaminergic and cholinergic phenotypes. Dexamethasone enhanced the effect of BaP on cell numbers and altered the impact on neurotransmitter phenotypes; whereas BaP alone shifted differentiation away from the cholinergic phenotype and toward the dopaminergic phenotype, in the presence of dexamethasone, it did the opposite. Chlorpyrifos coexposure augmented BaP inhibition of cell growth and enhanced the BaP-induced shift in phenotype toward a higher proportion of dopaminergic cells. Nicotine had no effect on BaP-induced changes in cell number or growth, but synergistically enhanced the BaP suppression of differentiation into both dopaminergic and cholinergic phenotypes equally.

**CONCLUSION:** Our results indicate that, although BaP can act directly as a developmental neurotoxicant, its impact is greatly modified by coexposure to other, commonly encountered neurotoxicants from prenatal drug therapy, pesticides, or tobacco. Accordingly, neurodevelopmental effects attributable to polycyclic aromatic hydrocarbons may be quite different depending on which other agents are present and their concentrations relative to each other.

## INTRODUCTION

Exposures to polycyclic aromatic hydrocarbons (PAHs) are ubiquitous, given their presence in combustion products of all kinds, including diesel exhaust, broiled foods, and smoke arising from wood, coal or tobacco. Although most studies of the adverse effects of PAHs center around their properties as carcinogens, recent reports indicate that these agents are also developmental neurotoxicants, potentially contributing to the explosive increase in the incidence of neurobehavioral disorders (Grandjean and Landrigan 2006). Epidemiological studies show a relationship between fetal exposure to PAHs, head circumference and cognitive performance (Perera et al. 2003; 2006) but only a limited number of animal studies have explored whether these agents directly affect brain development. Benzo[a]pyrene (BaP), a PAH prototype, produces behavioral deficits in adults (Saunders et al. 2002) and, with fetal or neonatal exposure, leads to persistent anomalies in cognitive performance, anxiety-related behaviors, hippocampal function and neurochemical indices of cerebral activity (Chen et al. 2012; Hood et al. 2000; Wormley et al. 2004). Importantly, BaP exposure of neuronal cells in culture interferes with neurodifferentiation (Brown et al. 2007; Slotkin and Seidler 2009), indicating that the PAHs act directly as developmental neurotoxicants, exclusive of endocrine disruption or other systemic effects in the maternal-fetal unit or the newborn. We recently showed that BaP slows the ability of differentiating neuronotypic cells to exit the mitotic cycle and to initiate neurodifferentiation, resulting in increases in cell number at the expense of cell growth, neurite formation and development of neurotransmitter phenotypes (Slotkin and Seidler 2009). Such direct interference with neural cell differentiation could readily explain the observed correlation of prenatal PAH exposures in humans to behavioral dysfunction (Perera et al. 2005, 2006).

One major difference between laboratory and human PAH studies is that, whereas basic research tends to focus on exposures to single agents, humans are simultaneously exposed to other developmental neurotoxicants along with the PAHs, such as tobacco smoke and pesticides (Perera et al. 2005). This paper examines whether the direct effects of PAHs on neuronal development are modified by simultaneous exposure to other commonly-encountered neurotoxicants. Specifically, we looked at the combination of BaP with a glucocorticoid (dexamethasone), an organophosphate pesticide (chlorpyrifos) and nicotine. Each of these secondary agents has been well-studied for developmental neurotoxicity *in vivo* and *in vitro*, and they all represent major human exposure hazards. Glucocorticoids are the consensus treatment for preterm labor occurring between 24 and 34 weeks of gestation in order to prevent respiratory distress syndrome (Gilstrap et al. 1995); currently, one of every ten newborns in the U.S. has undergone this treatment (Matthews et al. 2002). Organophosphates represent nearly 50% of worldwide insecticide use and exposure of the human population is virtually ubiquitous (Casida and Quistad 2004). Nicotine coexposure with PAHs is common because of the presence of both in cigarette smoke, whether from active maternal smoking, or from second- and third-hand exposure (Hoh et al. 2012; Perera et al. 2005).

For our studies, we utilized PC12 cells, a well-characterized model for neurodifferentiation (Teng and Greene 1994), with protocols established previously for characterizing developmental neurotoxicity (Qiao et al. 2001, 2003, 2005; Slotkin et al. 2007a, b, 2008; Song et al. 1998). Effects on cell number were determined by measuring DNA content, since each neuronotypic cell contains only a single nucleus (Winick and Noble 1965). Cell size and membrane outgrowth associated with the formation of neurites were assessed by measurements of cell proteins (total protein/DNA, membrane protein/DNA and membrane protein/total protein). Finally, we assayed

tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), the two enzymes that delineate differentiation into the dopaminergic (TH) and cholinergic (ChAT) phenotypes that are the distinctive fate of PC12 cells (Teng and Greene 1994).

## METHODS

*Cell cultures.* Because of the clonal instability of the PC12 cell line (Fujita et al. 1989), the experiments were performed on cells that had undergone fewer than five passages. As described previously (Qiao et al. 2003; Song et al. 1998), PC12 cells (American Type Culture Collection CRL-1721, obtained from the Duke Comprehensive Cancer Center, Durham, NC) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% horse serum (Sigma), 5% fetal bovine serum (Sigma), and 50 µg/ml penicillin streptomycin (Invitrogen, Carlsbad, CA). Incubations were carried out with 5% CO<sub>2</sub> at 37°C, standard conditions for PC12 cells. To initiate neurodifferentiation (Jameson et al. 2006b; Slotkin et al. 2007b; Teng and Greene 1994), the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (Promega Corporation, Madison, WI); each culture was examined under a microscope to verify the outgrowth of neurites.

Toxicant exposures were all commenced simultaneously with the addition of nerve growth factor, so as to be present throughout neurodifferentiation. For BaP (Sigma) exposure, we chose two concentrations based on earlier studies with this model (Slotkin and Seidler 2009), one just at the threshold for effects (1 µM) and a higher concentration showing robust effects (10 µM); these concentrations are similar to those shown in vivo to produce lasting neurochemical and neurobehavioral effects after early-life exposures (Brown et al. 2007; Chen et al. 2012). Concentrations for the other agents were similarly based on earlier work, and were chosen to be

just at the threshold for effects on cell number and growth, so as to allow detection of interactions with BaP (Abreu-Villaça et al. 2005; Jameson et al. 2006a; Qiao et al. 2003; Slotkin et al. 2012; Song et al. 1998): dexamethasone (0.1  $\mu$ M; Sigma), chlorpyrifos (30  $\mu$ M; Chem Service, West Chester, PA) and nicotine bitartrate (10  $\mu$ M; Sigma). For dexamethasone, we conducted additional studies at a higher concentration (1  $\mu$ M). Because of the limited water solubility of BaP and chlorpyrifos, these agents were dissolved in dimethylsulfoxide (Sigma; final concentration 0.1%), which was also added to all the samples regardless of treatment; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation (Qiao et al. 2001; Song et al. 1998). The medium was changed every 48 hr with the continued inclusion of nerve growth factor and test substances; assays were carried out after six days of exposure.

*Assays.* Cells were harvested, washed, and the DNA and protein fractions were isolated and analyzed as described previously (Slotkin et al. 2007b). Measurements of DNA, total protein and membrane protein were used as biomarkers for cell number, cell growth and neurite growth (Qiao et al. 2003; Song et al. 1998). Since the DNA per cell is constant, cell growth entails an obligatory increase in the total protein per cell (protein/DNA ratio) as well as membrane protein per cell (membrane protein/DNA ratio). If cell growth represents simply an increase in the perikaryal area, then the ratio of membrane to total protein would fall in parallel with the decline in the surface-to-volume ratio (volume increases with the cube of the perikaryal radius, whereas surface area increases with the square of the radius); however, when neurites are formed as a consequence of neurodifferentiation, this produces a specific *rise* in the ratio. Each of these biomarkers has been validated in prior studies by direct measurement of cell number (Powers et al. 2010; Roy et al. 2005), perikaryal area (Roy et al. 2005) and neurite formation (Das and Barone 1999; Howard et al. 2005; Song et al. 1998). To assess neurodifferentiation into

dopamine and acetylcholine phenotypes, we assayed the activities of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively, using established techniques (Jameson et al. 2006a, b).

*Data analysis.* Each study was performed using 2-5 separate batches of cells, with 3-4 independent cultures for each treatment in each batch; each batch of cells comprised a separately prepared, frozen and thawed passage. Results are presented as mean  $\pm$  SE, with treatment comparisons carried out by analysis of variance (ANOVA; data log-transformed when variance was heterogeneous or where comparisons were based on proportional changes) followed by Fisher's Protected Least Significant Difference Test for post-hoc comparisons of individual treatments. Each treatment paradigm involved an initial three-factor ANOVA: factor 1 = BaP concentration; factor 2 = concentration of second agent (dexamethasone, chlorpyrifos or nicotine); factor 3 = cell batch. In each case, we found that the treatment effects were the same across the different batches of cells, although the absolute values differed from batch to batch. Accordingly, we normalized the results across batches prior to combining them for presentation. Significance was assumed at  $p < 0.05$ .

The experimental design required two different ways of considering the treatment variables. To characterize the effects of BaP alone, the second agent alone, or the combined treatment versus controls or versus each other, all of the treatment groups were first considered as a one-dimensional factor in the statistical design. In this formulation, each treatment can be compared to the control group or to any of the other treatments. Then, to determine whether the effects of BaP and the second agent were interactive, the treatment factors were changed to a two-dimensional design (factor 1 = BaP, factor 2 = second agent). In this formulation, synergistic, less-than-additive or antagonistic effects would appear as significant interactions between the

two treatment dimensions, whereas simple, additive effects would not show significant interactions. For example, although the one-factor arrangement of the data can show that a combined exposure might be worse than either exposure alone, the two-factor arrangement enables us to determine whether the worsened effect represents the additive effects of the two agents, or whether the combination gives a response that is greater or lesser than the predicted, additive value.

## RESULTS

*BaP and dexamethasone.* In agreement with earlier results (Slotkin and Seidler 2009), exposure to BaP alone during neurodifferentiation produced an elevation in the total of number of cells, as monitored by DNA content (Figure 1A), at the expense of cell enlargement, as evidenced by decrements in the total protein/DNA ratio (Figure 1B) and membrane protein/DNA ratio (Figure 1C). BaP by itself did not produce a significant change in the membrane/total protein ratio (Figure 1D); since smaller cells have an elevated ratio, the lack of change in this parameter, combined with the drop in total protein/DNA (smaller cells), connotes interference with the neurite formation that accompanies neurodifferentiation (Slotkin and Seidler 2009). In the absence of BaP, dexamethasone produced a significant decrement in cell numbers (Figure 1A). Accordingly, if the two treatments were simply additive, dexamethasone would be expected to reduce the effect of BaP on DNA content. Instead, it augmented it. At the low dexamethasone concentration, 1  $\mu$ M BaP produced a significant increase in cell numbers, whereas the same concentration was ineffective in the absence of dexamethasone; the increase evoked by 10  $\mu$ M BaP in the presence of dexamethasone remained just as high as before; superimposed on the

reduced baseline values caused by dexamethasone alone, the effects of BaP were thus synergistically enhanced (Figure 1A, offset panel).

By itself, dexamethasone had the opposite effect on cell growth from that obtained with BaP, evidenced by an increase in total protein/DNA (Figure 1B). When the two treatments were combined, the net outcome reflected these opposing actions, representing simple additive effects (no interaction of BaP  $\times$  dexamethasone). Dexamethasone had little or no effect on the membrane protein/DNA ratio and again, the combination showed simple additive actions of the two agents (Figure 1C). Because dexamethasone increased cell size without changing the membrane protein concentration, the membrane/total protein ratio fell, reflecting impaired neurite formation (Figure 1D), in agreement with earlier findings (Jameson et al. 2006a); again, this effect showed simple additivity with BaP.

Individually, BaP and dexamethasone had opposite effects on TH activity (Figure 2A). By itself, BaP evoked a reduction in TH, whereas dexamethasone alone produced a substantial increase. In the presence of dexamethasone, BaP showed an enhanced ability to reduce TH, reflecting a synergistic interaction of the two treatments (Figure 2A, offset panel). A different pattern was seen for effects on ChAT activity (Figure 2B). BaP alone produced a large decrease, as did dexamethasone; the combined treatment also showed a decrement, but distinctly less than would be expected from simple additive effects of the two treatments (Figure 2B, offset panel). Accordingly, the phenotypic outcome, assessed by the TH/ChAT ratio, was completely reversed by the double treatment (Figure 2C). Either BaP or dexamethasone alone elevated the ratio, reflecting a shift from the cholinergic to the dopaminergic phenotype. However, in the presence of dexamethasone, BaP reduced the ratio, an effect opposite to that seen with BaP by itself (Figure 2C and offset panel).

*BaP and chlorpyrifos.* In contrast to the combination of BaP with dexamethasone, cotreatment of BaP and chlorpyrifos produced interactions primarily involving cell growth parameters rather than cell numbers. Chlorpyrifos did not alter the effect of BaP on DNA content (Figure 3A) but did shift the response of both the total protein/DNA ratio (Figure 3B) and membrane protein/DNA ratio (Figure 3C), enhancing the reduction caused by BaP (significant BaP  $\times$  chlorpyrifos interaction for both ratios). Significant enhancement of the BaP effect was obtained at either the low or high BaP concentration (Figure 3B, offset panel; Figure 3C offset panel). There was a small, but significant increment in the membrane/total protein ratio in all the chlorpyrifos groups, regardless of whether BaP was included, without any interaction between the two treatments (Figure 3D).

Chlorpyrifos altered the ability of BaP to suppress neurodifferentiation. For TH activity, the combined treatment showed a smaller BaP induced decrement than that obtained with BaP alone, reflected in a significant BaP  $\times$  chlorpyrifos interaction (Figure 4A and offset panel). By itself, chlorpyrifos reduced ChAT but the interaction with BaP was purely additive (no interaction), so that the net outcome was an even greater reduction in ChAT compared to that evoked by BaP or chlorpyrifos alone (Figure 4B). With the combined exposure, the smaller decrease in TH and greater deficit in ChAT, produced a larger elevation of the TH/ChAT ratio than that obtained with either treatment alone (Figure 4C), a synergistic enhancement that reflected greater-than-additive effects (Figure 4C, offset panel).

*BaP and nicotine.* In contrast to the other agents, 10  $\mu$ M nicotine did not alter the effect of BaP on any of the parameters of cell number or growth (Figure 5). Nevertheless, it did influence the phenotypic outcome. For TH activity, nicotine enhanced the suppression caused by BaP,

resulting in a larger deficit (Figure 6A); the effect showed a significant BaP  $\times$  nicotine interaction, indicating synergistic effects (Figure 6A, offset panel). The same pattern was seen for ChAT, namely an enhanced effect of BaP in the presence of nicotine, resulting in greater-than-additive reductions in activity (Figure 6B and offset panel). Because the synergistic effect of the combination was equivalent for both TH and ChAT, the shift toward the dopaminergic and away from the cholinergic phenotype (increased TH/ChAT ratio) was equivalent for BaP alone and for BaP in the presence of nicotine (Figure 6C).

## DISCUSSION

The major finding of this study is that the ability of BaP to interfere with neurodifferentiation is greatly modified by coexposure to other common toxicants. For some endpoints, the alterations of BaP effects caused by the secondary agent are in opposite directions from each other. This implies that, for environmental exposures, the observed outcome for BaP is likely to depend on the nature and concentration of other neurotoxicants to which the fetus or neonate has been exposed.

By itself, BaP produced effects entirely consistent with impaired neurodifferentiation (Slotkin and Seidler 2009). Upon addition of nerve growth factor, PC12 cells begin to exit the mitotic cycle and differentiate into dopaminergic and cholinergic neuronal phenotypes (Teng and Greene 1994). BaP prolongs the period of mitotic activity, resulting in elevated cell numbers, at the expense of cell growth and differentiation (Slotkin and Seidler 2009); here, this was shown by an elevation in DNA content (more cells), along with reductions in indices of cell enlargement, neurite formation and emergence of neurotransmitter phenotypes, with the latter showing a greater impairment for acetylcholine (ChAT) than for dopamine (TH). Coexposure with

dexamethasone enhanced the effects of BaP. Although dexamethasone by itself reduced DNA content, the addition of BaP produced a synergistic increase over the baseline effect of dexamethasone, proportionally larger than that seen with BaP alone. Even more strikingly, with dexamethasone coexposure, BaP showed greater suppression of TH activity, whereas it had a lesser effect on ChAT. Thus, the net consequence of the combined exposure to BaP and dexamethasone was to reverse the impact of BaP on phenotype: BaP alone increased the TH/ChAT ratio, but in cells treated with dexamethasone, BaP decreased the ratio. Accordingly, dexamethasone completely shifted the impact of BaP on neurodifferentiation.

Although chlorpyrifos likewise altered the response to BaP, the outcomes were entirely different from those seen with dexamethasone. Rather than augmenting the effects on cell number, chlorpyrifos enhanced BaP inhibition of cell growth. At the same time, chlorpyrifos *reduced* the ability of BaP to impair the emergence of TH; consequently the impact on neuronal differentiation was to promote the dopaminergic phenotype at the expense of the cholinergic phenotype, to an even greater extent than was seen with either agent alone. This produced an even greater increase in the TH/ChAT ratio, exactly the opposite outcome from that seen when BaP was combined with dexamethasone. It is also notable that these combined effects of chlorpyrifos and BaP, reflect direct targeting of neurodifferentiation, rather than effects secondary to cholinesterase inhibition; PAHs and chlorpyrifos show additive inhibitory effects on cholinesterase (Jett et al. 1999), whereas we found that chlorpyrifos interfered with the effect of BaP on TH.

Nicotine coexposure produced yet a third set of outcomes for the effects of BaP on neurodifferentiation. Nicotine did not affect the ability of BaP to increase cell numbers or impair growth parameters but it produced synergistic effects on suppression of both the dopaminergic

and cholinergic phenotypes. In this case, the interaction was equally targeted toward TH and ChAT, so that, although both neurotransmitter subtypes showed deficits, there was no further shift in phenotypic preference from the combined exposure as compared to BaP alone.

There are obvious limitations inherent in any in vitro model of developmental neurotoxicity, as detailed previously (Coecke et al. 2007; Qiao et al. 2001; Song et al. 1998) but it is worth repeating the major points here. The main purpose of in vitro models is to assess direct effects of toxicants, allowing for dissection of cause-and-effect relationships that cannot readily be studied in vivo. The first limitation, then, is that cell culture models lack the ability to detect more complex neurodevelopmental events involved in brain assembly, including cell-to-cell interactions and architectural modeling of brain regions. Second, in vitro exposures typically involve treatments over a period of hours, whereas in vivo exposures encompass much more extended exposure periods. Third, transformed cell lines such as PC12 cells, usually are less sensitive to toxicants than are primary neurons. All these factors mean that it is difficult to extrapolate relevant in vivo concentrations of toxicants from in vitro results alone, and typically, the concentrations required for a given effect in vitro will be substantially higher than those required for parallel effects in vivo (Coecke et al. 2007). Nevertheless, it should be noted that the BaP concentrations used here do correspond to doses required for adverse effects in developing rats (Brown et al. 2007; Chen et al. 2012). In the present work, we chose the PC12 line for specific reasons. The primary effect of BaP is to delay the transition from cell replication to neurodifferentiation (Slotkin and Seidler 2009). Primary neurons do not divide in culture and are in heterogeneous states of neurodifferentiation, whereas PC12 cells undergo uniform differentiation triggered by addition of nerve growth factor. Thus, primary neurons are problematic for these assessments, whereas the PC12 line is especially useful (Coecke et al.

2007; Radio et al. 2008). Similarly, because the PC12 line has two defined differentiation endpoints (acetylcholine, dopamine), it can be readily used to evaluate the potential of test agents to interfere with the appearance of neurotransmitter phenotypes, providing a proof-of-principle for neurotransmitter switching; this leads the way to examining a wider range of phenotypes in vivo.

## CONCLUSIONS

Although PAHs can act directly as developmental neurotoxicants, exclusive of endocrine or secondary systemic effects, their impact is greatly modified by coexposure to other neurotoxicants. Accordingly, the effects attributable to PAH may be quite different depending on which other agents are present and their concentrations relative to each other. Studies of human populations may thus show different outcomes for PAH effects depending on these other contributors. Here, we selected specific contaminants (dexamethasone, chlorpyrifos, nicotine) that can be tracked from medical histories or from environmental exposure assessments, an approach that points the way to being able to study these interactions in human populations using existing databases. Indeed, our results reinforce the value of in vitro models in evaluating the complex effects of multiple toxicant exposures, and in producing testable hypotheses for clinical and epidemiologic studies of human populations.

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## FIGURE LEGENDS

Figure 1. Effects of BaP in combination with dexamethasone (Dex) on indices of cell number and cell growth: (A) DNA, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane protein/total protein. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVAs for the main effects of treatment and interactions of BaP with dexamethasone are shown at the top of each panel; where there was a significant interaction, the offset panel shows the percent change caused by BaP relative to the corresponding BaP 0 group for each dexamethasone concentration. Note that we did not evaluate the combination of 1  $\mu$ M BaP and 1  $\mu$ M dexamethasone. Asterisks denote groups that are statistically significant from the corresponding BaP 0 group; daggers denote significance from the corresponding Dex 0 group. NS, not significant.

Figure 2. Effects of BaP in combination with dexamethasone (Dex) on neurodifferentiation into dopaminergic and cholinergic phenotypes: (A) Tyrosine hydroxylase, (B) Choline acetyltransferase, (C) TH/ChAT ratio. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVAs for the main effects of treatment and interactions of BaP with dexamethasone are shown at the top of each panel; the offset panels show the percent change caused by BaP relative to the corresponding BaP 0 group for each dexamethasone concentration. Asterisks denote groups that are statistically significant from the corresponding BaP 0 group; daggers denote significance from the corresponding Dex 0 group. NS, not significant.

Figure 3. Effects of BaP in combination with chlorpyrifos (CPF) on indices of cell number and cell growth: (A) DNA, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D)

membrane protein/total protein. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVAs for the main effects of treatment and interactions of BaP with chlorpyrifos are shown at the top of each panel; where there was a significant interaction, the offset panel shows the percent change caused by BaP relative to the corresponding BaP 0 group for each chlorpyrifos concentration. Asterisks denote groups that are statistically significant from the corresponding BaP 0 group; daggers denote significance from the corresponding CPF 0 group. NS, not significant.

Figure 4. Effects of BaP in combination with chlorpyrifos (CPF) on neurodifferentiation into dopaminergic and cholinergic phenotypes: (A) Tyrosine hydroxylase, (B) Choline acetyltransferase, (C) TH/ChAT ratio. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVAs for the main effects of treatment and interactions of BaP with chlorpyrifos are shown at the top of each panel; where there was a significant interaction, the offset panel shows the percent change caused by BaP relative to the corresponding BaP 0 group for each chlorpyrifos concentration. Asterisks denote groups that are statistically significant from the corresponding BaP 0 group; daggers denote significance from the corresponding CPF 0 group. NS, not significant.

Figure 5. Effects of BaP in combination with nicotine (Nic) on indices of cell number and cell growth: (A) DNA, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane protein/total protein. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVAs for the main effects of treatment and interactions of BaP with nicotine are shown at the top of each panel. Asterisks denote groups that are statistically significant from the corresponding BaP 0 group; dagger denotes significance from the corresponding Nic 0 group. NS, not significant.

Figure 6. Effects of BaP in combination with nicotine (Nic) on neurodifferentiation into dopaminergic and cholinergic phenotypes: (A) Tyrosine hydroxylase, (B) Choline acetyltransferase, (C) TH/ChAT ratio. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVAs for the main effects of treatment and interactions of BaP with nicotine are shown at the top of each panel; where there was a significant interaction, the offset panel shows the percent change caused by BaP relative to the corresponding BaP 0 group for each nicotine concentration. Asterisks denote groups that are statistically significant from the corresponding BaP 0 group; daggers denote significance from the corresponding Nic 0 group. NS, not significant.











